Journal of Horticultural Science & Biotechnology (2004) **79** (2) 175–181

Soil and foliar nitrogen supply affects the composition of nitrogen and carbohydrates in young almond trees

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SUMMARY

June-budded 'Nonpareil/Nemaguard' almond (*Prunus dulcis* (Mill) D. A. Webb) trees were fertigated with one of five nitrogen (N) concentrations (0, 5, 10, 15, or 20 mM) in a modified Hoagland's solution from July to September. In October, the trees were sprayed twice with either water or 3% urea, then harvested after natural leaf fall and stored at 2°C. Trees were destructively sampled during winter storage to determine their concentrations of amino acids, protein, and non-structural carbohydrates (TNC). Increasing N supply either via N fertigation during the growing season or with foliar urea applications in the fall increased the concentrations of both free and total amino acids, but decreased their C/N ratios. Moreover, as the N supply increased, the proportion of nitrogen stored as free amino acids also increased. However, protein was still the main form of N used for storage. The predominant amino acid in both the free and the total amino-acid pools was arginine. Arginine N accounted for an increasing proportion of the total N in both the free and the total amino acids as the nitrogen supply was increased. However, the proportion of arginine N was higher in the free amino acids than in the total amino acids. A negative relationship was found between total amino acid and non-structural carbohydrate concentrations, suggesting that TNC is increasingly used for N assimilation as the supply of nitrogen increases. Urea applications decreased the concentrations of glucose, fructose, and sucrose, but had little influence on concentrations of sorbitol and starch. We conclude that protein is the primary form of storage N, and that arginine is the predominant amino acid. Furthermore, the synthesis of amino acids and proteins comes at the expense of non-structural carbohydrates.

Deciduous fruit trees store nitrogen and carbohydrates in the previous year, and then remobilize these accumulated compounds for new growth in the following growing season. This internal cycling provides the structural components and energy needed for early new growth (Taylor and May, 1967; Titus and Kang, 1982; Tromp, 1983; Oliveira and Priestley, 1988; Millard and Thomson, 1989; Loescher *et al*., 1990; Millard, 1995, 1996; Neilsen *et al*., 2001; Cheng and Fuchigami, 2002; Bi *et al*., 2003).

Non-structural carbohydrates include an insoluble starch fraction and a soluble sugar fraction, i.e., sucrose, glucose, fructose, and sorbitol (Oliveira and Priestley, 1988). Similar to stored carbohydrates, N reserves are composed of a soluble fraction that includes amino acids and amides, as well as an insoluble protein fraction (Taylor, 1967; Oliveira and Priestley, 1988). It is somewhat controversial as to whether proteins or free amino acids serve as the main storage form for nitrogen. Previous research with apple (Oland, 1959), peach (Taylor and May, 1967), and pear (Taylor *et al*., 1975) has shown that storage organs and dormant vegetative tissues contain a high proportion of their N in soluble forms, mainly free amino acids, with only a low proportion in proteins. Based on those studies, free amino acids would appear to be the primary source of nitrogenous reserves. However, more evidence suggests that protein may be the main form of N storage in the dormant tissues of apple and poplar trees (Tromp, 1970; Tromp and Ovaa, 1971; Shim *et al*., 1973; O'Kennedy *et al*., 1975; Kang and Titus, 1980; Titus and Kang, 1982; Kang *et al*., 1982; Coleman *et al*., 1991). Nevertheless, no tests had yet been reported on identifying the main form of storage N (protein v free amino acid) in young almond trees.

Arginine is, theoretically, the most efficient form of storage N because of its low C/N ratio (Titus and Kang, 1982). Free arginine has been found to be the principal constituent of extracts from dormant apple (Oland, 1959; O'Kennedy *et al*., 1975), peach (Taylor and May, 1967; Taylor and van den Ende, 1969), and poplar (Sagisaka, 1974). It is also the predominant amino acid in the proteins of apple trees with high levels of stored nitrogen (Tromp and Ovaa, 1973). Glutamine and asparagine are other important N constituents measured in dormant apple (Oland, 1959), but not in peach (Taylor and May, 1967). Although the level of arginine in woody tissues of dormant trees is considered the most sensitive indicator of tree-N status (Taylor and May, 1967), it is not known how the pool of amino acids in storage responds to different nitrogen supply in young almond trees. Therefore, the objectives of this study were to determine (1) the chemical composition of nitrogen and non-structural carbohydrates in response to different N supplies, and (2) the interaction between storage N and non-structural carbohydrates in young almond trees.

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MATERIALS AND METHODS

Experimental design

June-budded 'Nonpareil' almond (*Prunus dulcis* (Mill) D. A. Webb) trees, on 'Nemaguard' rootstocks, were planted in 7.6 l pots containing a 1:2:1 (v/v/v) mix of peat moss, pumice, and sandy loam soil. Trees were then grown under natural conditions in Corvallis, Oregon (44° 30' N, 123° 17' W). Starting from budbreak, the trees were fertigated every two weeks with 150 mg l^{-1} N, using Plantex® 20N-10P_2O_5-20K_2O water-soluble fertilizer with micronutrients (Plantex Corp., Ontario, Canada). On 1 July, the trees were selected for uniformity. Thirty plants were randomly assigned to one of five groups. From 1 July to 1 September, each group was fertigated twice weekly (300 ml per pot) with one of five N concentrations (0, 5, 10, 15, or 20 mM N from NH_4NO_3), using a modified Hoagland's solution (Hoagland and Arnon, 1950).

Fifteen plants from each N fertigation concentration were randomly selected and sprayed with 3% urea on 10 and 20 October, (F+U treatment). The remaining plants were sprayed only with water, as our control (F treatment). After natural leaf fall, all the trees were harvested bare-root in December and stored in a cold room at 2°C. Five plants from each treatment were destructively sampled and their stems and root systems were washed with double distilled water to remove any urea residue. All the samples were immediately put into a -80°C freezer for pre-freezing, then freeze-dried and ground to pass a 40-mesh screen.

Carbohydrates and nitrogen analysis

The composition and concentration of non-structural carbohydrates was determined via high performance liquid chromatography. Tissue samples (50 mg) were weighed and extracted three times at 70°C with 3 ml 80% ethanol. Xylitol was added as an internal standard, and the suspensions were centrifuged at 4000 **g** for 10 min. The extract was passed through ion exchange columns consisting of 1 ml Amberlite IRA-67 (acetate form) (Sigma) and 1 ml Dowex 50W (hydrogen form) (Sigma), then evaporated to dryness at 55°C, and dissolved in 10 ml water. After the appropriate dilution, 25 µl of the extract was injected into a Dionex DX-500 series chromatograph that was equipped with a Carbopac PA-1 column, a pulsed amperometric detector, and a gold electrode (Dionex, Sunnyvale, CA, USA). Carbohydrates were eluted at a flow rate of 1.0 ml min^{-1} with 200 mM NaOH for 15 min. The peak area and the calibration curve derived from the corresponding standard authentic sugar were used to determine individual sugar concentrations. Tissue residue, used for measuring starch content, was dried and digested with amyloglucosidase at 55°C overnight to convert starch to glucose. The concentration of glucose was quantified via the Dionex chromatograph.

To determine the concentration of total amino acids in our samples, 100 mg of tissue was weighed and hydrolyzed in 10 ml 6M hydrochloric acid at 110°C for 22 h (Tromp and Ovaa, 1973). Standard amino acids were added to the sample at the beginning. After cooling and filtration, the volume was brought to 25 ml, an aliquot of which was taken to remove HC1, then dissolved in a citrate buffer (pH 2.2). After proper dilution, 50 µl of the extract was injected into a Beckman 121 automatic amino-acid analyzer, equipped with an FR-10 spherical cation exchange resin column (Beckman Instruments, Inc., Fullerton, CA, USA). To assess the composition and concentration of free amino acids, 200 mg of tissue was weighed and extracted with 20 ml 80% ethanol at room temperature for 24 h. Standard amino acids were added to the sample before the extraction began. The extract was evaporated at 75 °C to dryness after filtration, and was dissolved in a citrate buffer (pH 2.2). The analysis then continued as described above.

Total amino acids were defined as the amino acids present in the samples after protein hydrolysis, and included both free amino acids and protein amino acids. The nitrogen in free amino acids or total amino acids was

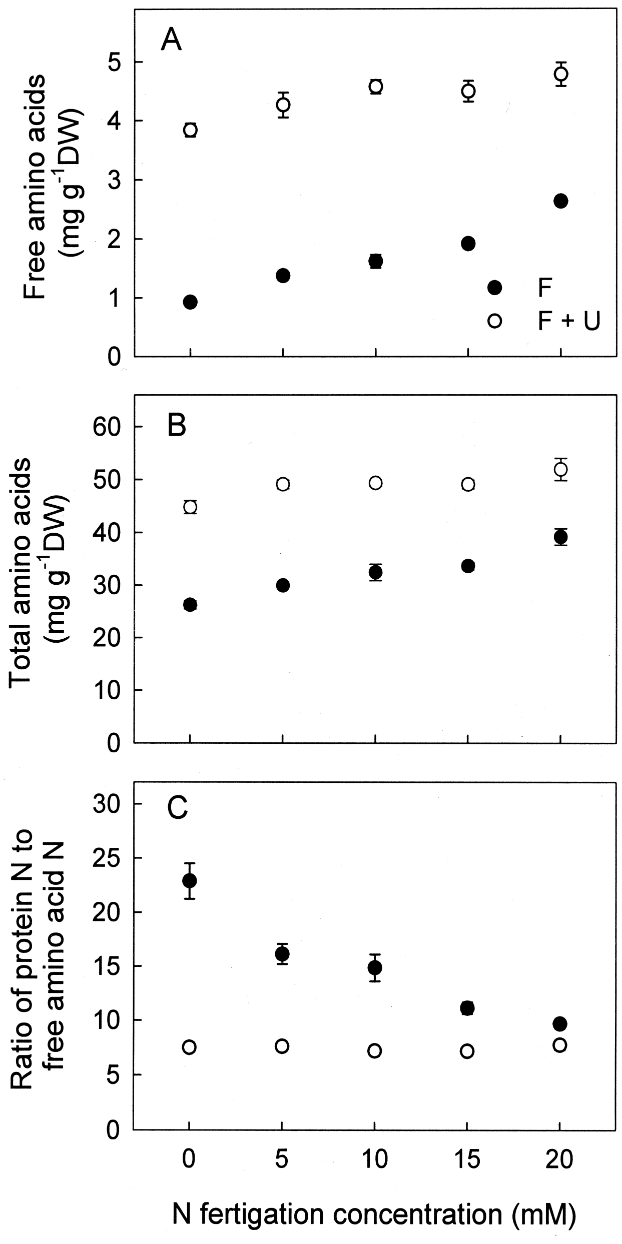

FIG.1
Effects of nitrogen [N] fertigation during the growing season and foliar urea application in the fall on concentrations of free (A) and total amino acids (B), and the ratio of protein N to free amino acid N (C) of young almond trees. Each value is the mean of five replicates. Error bars represent standard errors of the mean for each treatment. F = N fertigation, and F + U = N fertigation and foliar urea treatments.

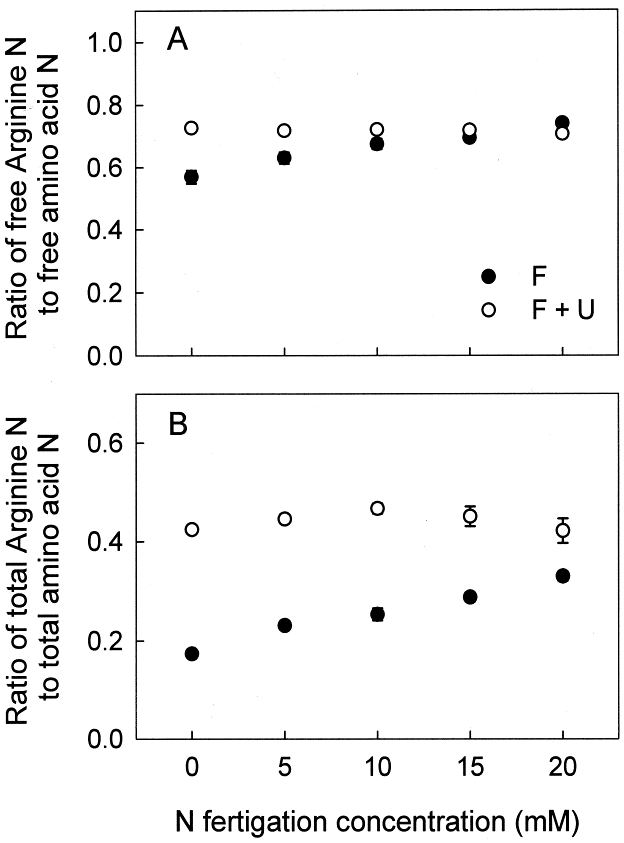

FIG.2
Effects of nitrogen [N] fertigation during the growing season and foliar urea application in the fall on the ratio of nitrogen in free arginine to that in free amino acids (A), and the ratio of nitrogen in total arginine to that in total amino acids (B) of young almond trees. Each value is the mean of five replicates. Error bars represent the standard errors of the mean for each treatment. F = N fertigation, and F+U = N fertigation and foliar urea treatments.

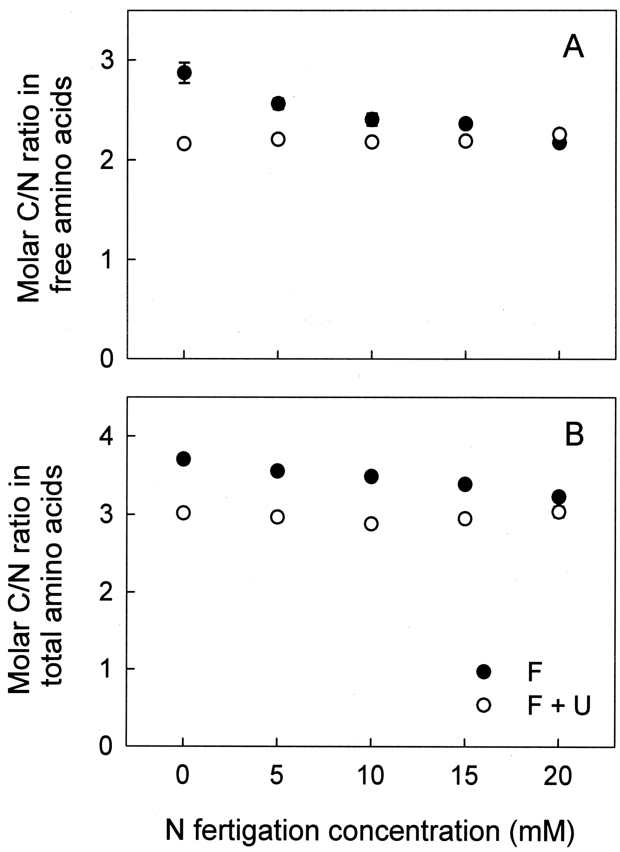

FIG.3
Effects of nitrogen [N] fertigation during the growing season and foliar urea application in the fall on molar Carbon/Nitrogen [C/N] ratio in free (A) and total amino acids (B) from young almond trees. Each value is the mean of five replicates. Error bars represent the standard errors of the mean for each treatment. F = N fertigation, and F+U = N fertigation and foliar urea treatments.

the sum of N from each individual amino acid. Furthermore, carbon in the free or the total amino acids was the sum of C from each individual amino acid. Carbon in the non-structural carbohydrates was the total of carbon in glucose, fructose, sucrose, sorbitol and starch. The sum of the soluble sugars and starch was considered the amount of total non-structural carbohydrates (TNC).

Statistical analysis

This experiment was a completely randomized design, with five replicates in each treatment. The amino-acid and carbohydrate data were analysed using analysis of variance (ANOVA). Comparisons of means among treatments were performed by contrasts, adjusting for multiple comparisons using Tukey's method. The relationship between total amino acid and TNC concentrations was determined by linear regression analysis. All statistical analyses were conducted with SAS (SAS Inst. Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Free amino acids and total amino acids

The concentration of free amino acids in fertigated almond trees (F) increased with increasing N-fertigation concentration (Figure 1A). This was also true for the concentration of total amino acids (Figure 1B). Applying foliar urea in the fall significantly increased (P<0.0001) the concentrations of both free and total amino acids at each given N-fertigation concentration. However, the concentrations of free amino acids and total amino acids in plants fertigated with lower N concentrations showed a greater response to urea treatment than those fertigated with higher amounts of nitrogen. Foliar urea applications increased the concentrations of free amino acids by 2.139 (20 mM) to 2.916 mg g^{-1}DW (0 mM), as well as the concentrations of total amino acids by 12.720 (20 mM) to 18.530 mg g^{-1}DW (0 mM). The ratio of protein N to free amino-acid N decreased with increasing N-fertigation concentrations when trees did not receive foliar urea (Figure 1C). Foliar urea application in the fall significantly decreased (P<0.000l) the N ratio at each given fertigation concentration, with trees that received less nitrogen being more responsive. All the trees treated with foliar urea had N ratios of approximately 7.5.

Our data clearly demonstrate that the amino acid concentrations in young almond trees are closely related to N supply. Increasing N supply by N- fertigation during the growing season or applying foliar urea in the fall increased the concentrations of both free and total amino acids during winter storage. The proportion

TABLE I

Effects of nitrogen (N) fertigation during the growing season and foliar urea application in the fall on the concentration of free amino acids in young almond trees

Amino acid	Free amino acid concentrations (mg g^{-1} DW)									
	Fertigation concentration (mM)									
	0		5		10		15		20	
	F[z]	F+U	F	F+U	F	F+U	F	F+U	F	F+U
Arg	0.279[y]	1.910	0.534	2.132	0.735	2.279	0.887	2.289	1.409	2.323
Glu	0.202	0.270	0.252	0.369	0.209	0.372	0.259	0.400	0.307	0.377
Pro	0.144	0.438	0.203	0.498	0.218	0.523	0.249	0.474	0.309	0.573
Asp	0.078	0.202	0.094	0.205	0.108	0.215	0.099	0.203	0.145	0.206
Ser	0.056	0.548	0.083	0.556	0.101	0.589	0.093	0.598	0.127	0.586
Ala	0.049	0.096	0.062	0.141	0.066	0.139	0.083	0.137	0.084	0.148
Phe	0.024	0.035	0.025	0.033	0.026	0.033	0.027	0.031	0.021	0.040
Met	0.020	0.016	0.025	0.019	0.024	0.016	0.045	0.020	0.033	0.031
Lys	0.016	0.142	0.032	0.144	0.056	0.170	0.071	0.240	0.099	0.261
Tyr	0.015	0.047	0.015	0.056	0.021	0.031	0.025	0.050	0.022	0.074
Ile	0.011	0.011	0.013	0.021	0.019	0.028	0.023	0.053	0.019	0.089
Val	0.008	0.005	0.012	0.014	0.009	0.006	0.011	0.011	0.014	0.024
His	0.006	0.027	0.011	0.039	0.015	0.053	0.018	0.048	0.032	0.052
Leu	0.006	0.028	0.009	0.034	0.012	0.033	0.017	0.033	0.015	0.045
Gly	0.006	0.009	0.007	0.011	0.007	0.011	0.009	0.013	0.009	0.014
Cyr	0.005	0.005	0.006	0.004	0.006	0.004	0.006	0.006	0.006	0.008

[z]F = N fertigation and F+U = N fertigation and foliar urea treatments.
[y]Each value is the mean of five replicates.

of N stored as free amino acids was also increased when the nitrogen supply was increased by either fertigation or urea. However, protein was still the main form of stored N in our almond trees (Figure 1C). These results agree with those reported for apple (Tromp, 1970; O'Kennedy *et al*., 1975; Kang and Titus, 1980) and poplar (Coleman *et al*., 1991). In contrast, other research has shown that soluble compounds, including amino acids, are the main storage form of N for apple (Oland, 1959), peach (Taylor and May, 1967), and pear (Taylor *et al*., 1975). This difference may be a consequence of different sampling times and protocols used to extract the various nitrogenous reserves. Protein hydrolysis begins a few weeks before budbreak (Oland, 1959; Hill-Cottingham, 1968; Tromp, 1970; O'Kennedy *et al*., 1975), resulting in a rapid increase in the level of soluble nitrogen available for plant growth (Kang *et al*., 1982). If the samples were collected close to budbreak, the concentration of non-protein N could increase markedly because of protein hydrolysis.

TABLE II

Effects of nitrogen (N) fertigation during the growing season and foliar urea application in the fall on the concentration of total ammo acids in young almond trees

Amino acid	Total amino acid concentrations (mg g^{-1} DW)									
	Fertigation concentration (mM)									
	0		5		10		15		20	
	F[z]	F+U	F	F+U	F	F+U	F	F+U	F	F+U
Arg	2.082[y]	10.106	3.188	11.802	3.911	12.983	4.469	11.988	6.399	11.880
Glu	3.530	4.766	3.920	5.075	4.058	5.027	4.052	5.081	4.614	5.797
Pro	1.648	2.332	1.883	2.657	1.902	2.662	1.954	2.484	2.153	3.153
Asp	2.950	6.681	3.261	6.568	3.381	6.679	3.310	6.825	3.947	6.800
Ser	1.514	1.836	1.621	2.065	1.659	2.039	1.635	2.106	1.893	2.176
Ala	1.495	1.854	1.602	2.070	1.625	2.006	1.661	2.031	1.809	2.663
Phe	1.204	1.684	1.410	1.835	1.406	1.688	1.479	1.802	1.609	1.898
Met	0.451	0.487	0.453	0.514	0.456	0.512	0.525	0.562	0.431	0.625
Lys	1.995	2.835	2.154	3.082	2.269	2.995	2.273	3.042	2.698	3.117
Tyr	0.776	1.135	0.917	1.236	0.959	1.129	1.028	1.238	1.121	1.314
Ile	1.220	1.497	1.345	1.643	1.342	1.544	1.347	1.577	1.467	1.738
Val	1.628	2.003	1.746	2.318	1.813	2.193	1.916	2.208	1.919	2.481
His	0.912	1.331	1.029	1.441	1.109	1.414	1.108	1.485	1.399	1.534
Leu	2.099	2.709	2.351	2.939	2.404	2.793	2.433	2.957	2.716	3.163
Gly	1.372	1.709	1.507	1.904	1.533	1.812	1.558	1.863	1.754	2.169
Cyr	0.054	0.095	0.078	0.081	0.098	0.109	0.104	0.113	0.116	0.134

[z]F = N fertigation and F+U = N fertigation and foliar urea treatments.
[y]Each value is the mean of five replicates.

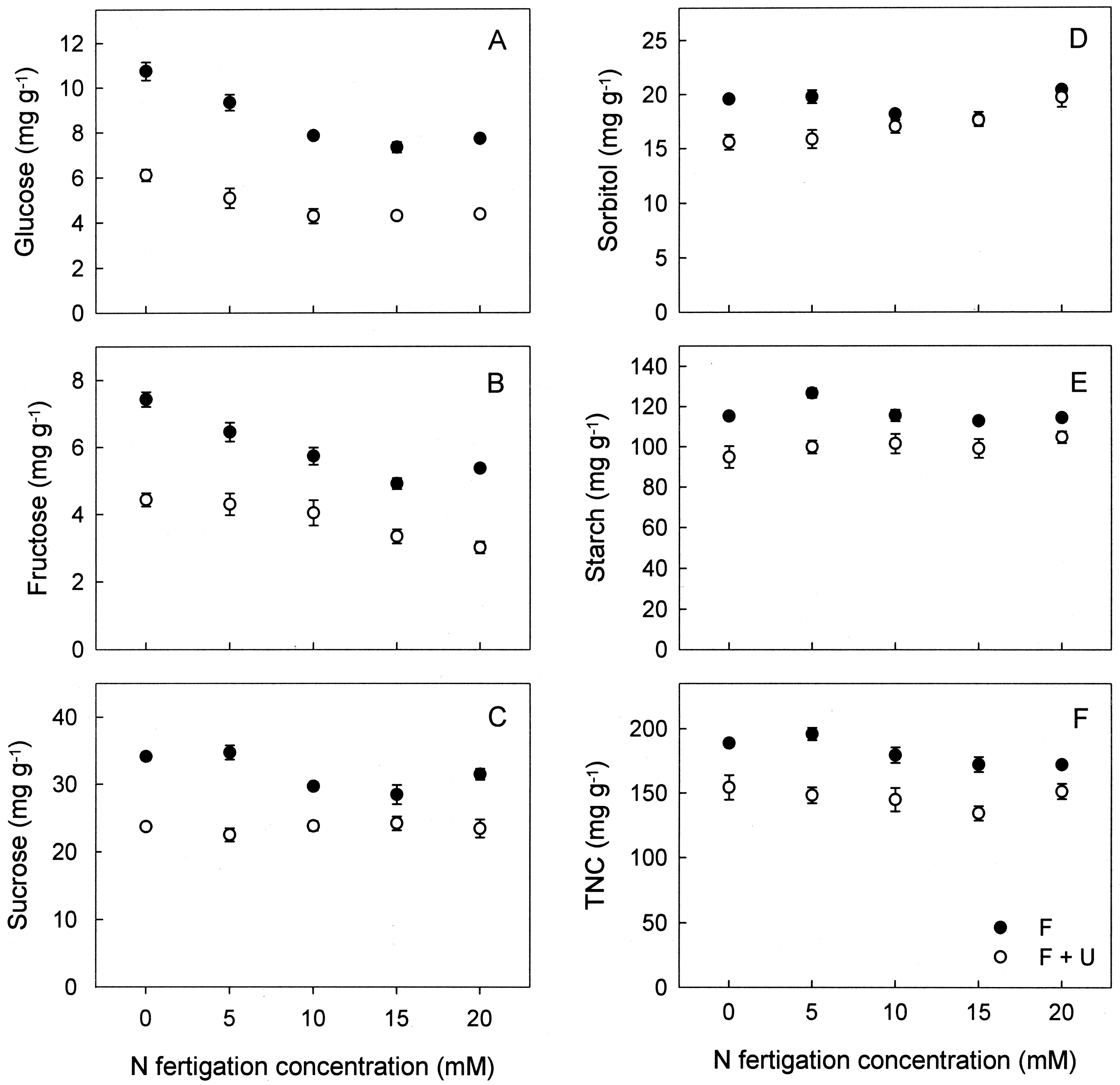

FIG. 4
Effects of nitrogen [N] fertigation during the growing season and foliar urea application in the fall on the concentrations (mg g^{-1}DW) of glucose (A), fructose (B), sucrose (C), sorbitol (D), starch (E), and TNC [total non-structural carbohydrates] (F) from young almond trees. Each value is the mean of five replicates. Error bars represent the standard errors of the mean for each treatment. F = N fertigation, and F+U = N fertigation and foliar urea treatments.

Individual amino acids

On a whole-tree basis, arginine (Arg), glutamate (Glu), proline (Pro), aspartate (Asp), serine (Ser), alanine (Ala), and lysine (Lys) were identified as the main constituents of storage nitrogen in both free and total amino acids; their concentrations increased with the increasing N-fertigation concentrations (Tables I, II). Foliar urea applications generally increased the concentrations of individual amino acids, especially for Arg (Tables I, II), which was the predominant amino acid in both free amino-acid and total amino-acid pools.

The ratio of N in free Arg to that in the free amino acids increased with increasing N-fertigation concentration in trees not receiving foliar urea (Figure 2A). Foliar urea application significantly increased (P<0.01) the N ratio of free Arg to free amino acids in trees fertigated with 0 and 5 mM N concentrations. All trees treated with foliar urea had N ratios of approximately 0.7. The ratio of N in total Arg to that in total amino acids also increased with increasing N-fertigation concentrations in trees not receiving the fall urea application (Figure 2B). Foliar urea significantly increased (P<0.0001) the N ratio at each given level of fertigation, but trees fertigated at low-N concentrations responded more to urea treatment than those receiving higher nitrogen concentrations. All trees treated with foliar urea had N ratios of approximately 0.4.

Our results demonstrate that, in dormant almond trees, Arg is the main amino acid in both the free and the total amino-acid pools. The N found in Arg accounted for

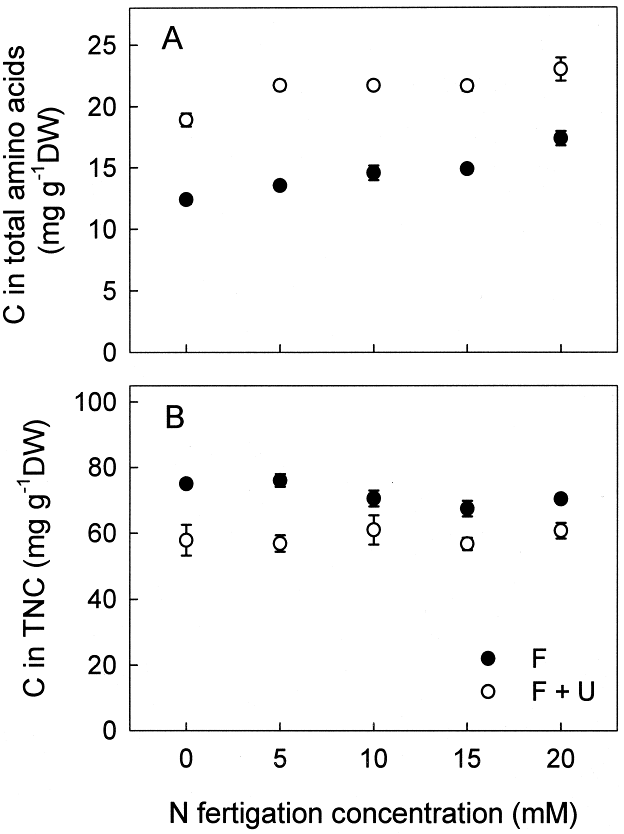

FIG. 5
Effects of nitrogen [N] fertigation during the growing season and foliar urea application in the fall on carbon concentrations [C] in total amino acids (A) and in total non-structural carbohydrates [TNC] (B) from young almond trees. Each value is the mean of five replicates. Error bars represent the standard errors of the mean for each treatment. F = N fertigation, and F+U = N fertigation and foliar urea treatments.

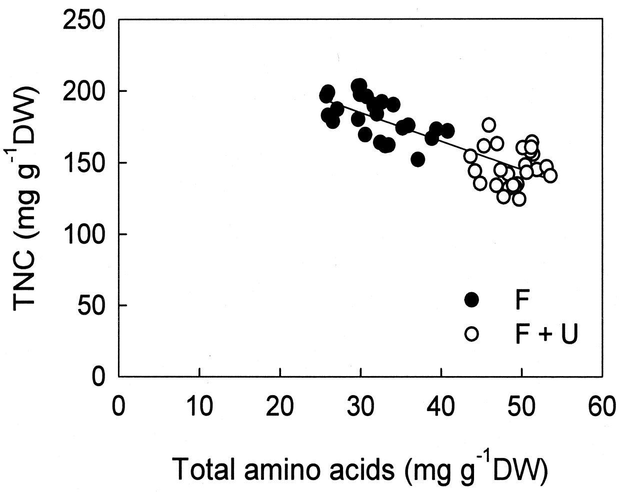

FIG. 6
Concentrations of non-structural carbohydrates (TNC) in relation to concentrations of total amino acids from young almond trees. Regression equation: Y = 243.58 - 1.964X (r^2 = 0.66, P<0.0001). F = N fertigation, and F+U = N fertigation and foliar urea treatments.

an increasing proportion of the nitrogen in the free and total amino acids as the N supply increased. This agrees, in part, with previous findings with apple (Oland, 1959; Tromp and Ovaa, 1973; O'Kennedy *et al*., 1975) and peach (Taylor and May, 1967; Taylor and van den Ende, 1969). The proportion of arginine N in the free amino acids was also higher than that measured in the total amino acids. We also noted that Glu and Asp served as important constituents of stored N.

C/N ratio

The C/N ratios of free amino acids and total amino acids decreased with increasing N-fertigation concentrations when trees did not receive foliar urea in the fall (Figure 3A, B). As the supply of nitrogen increased, N-rich amino acids, e.g., Arg, accounted for an increasing proportion of the free and the total amino acids (Tables I, II; see also Tromp and Ovaa, 1973). This resulted in lower C/N ratios for both free and total amino-acid pools.

Applying foliar urea in the fall significantly decreased (P<0.0001) the C/N ratio of free amino acids in trees fertigated with 0 mM to 15 mM N concentrations, but not the highest nitrogen concentration (20 mM). The C/N ratio in free amino acids was approximately 2.2 for all our urea-treated trees.

Fall applications of foliar urea also significantly decreased (P<0.0001) the C/N ratio in total amino acids at each given fertigation concentration. Compared with the ratio determined for the total amino acids (i.e., 3.0), free amino acids had a lower C/N ratio at each given fertigation concentration. This suggests that the proportion of N-rich amino acids, such as Arg, is higher in the free amino-acid pool than in the total amino-acid pool. That lower C/N ratio makes N storage more efficient in terms of carbon investment (Titus andKang, 1982).

Non-structural carbohydrates

Concentrations of glucose, fructose, and sucrose generally decreased with increasing N-fertigation concentrations, up to 15 mM N (Figure 4A, B, C). Foliar urea applications also significantly decreased (P<0.0001) their concentrations at all fertigation concentrations. The influence of urea was largest in trees that received the lowest concentration of nitrogen. In contrast, urea treatment significantly decreased (P<0.01) the amount of sorbitol only at the lowest two fertigation concentrations (0 and 5 mM N; Figure 4D). Starch concentration was slightly higher in trees fertigated at the lowest N concentrations (Figure 4E). Foliar urea also significantly decreased (P<0.0001) starch concentration at each given fertigation concentration.

Total non-structural carbohydrates (TNC) showed a response similar to that of starch with the N-fertigation treatments (Figure 4F). Foliar urea application in the fall significantly decreased (P<0.0001) TNC concentration at each fertigation concentration. These carbohydrates provide energy as well as carbon skeleton for N assimilation and the synthesis of amino acids and proteins (Oliveira and Priestley, 1988). In our study, the concentrations of all TNC components decreased when the N supply was augmented through foliar urea applications. This indicates an increased use of TNC for nitrogen assimilation as the N supply increases. The greater decrease in glucose, fructose, and sucrose concentrations (compared with sorbitol and starch) in response to urea suggests that those first three components are more readily available for N assimilation than the latter two. This can be explained in part by the fact that sorbitol and starch must first be converted to fructose or glucose before being usable.

Interaction between nitrogen and carbohydrates

The concentration of carbon (C) in the total amino acids increased with increasing fertigation concentrations (Figure 5A). In contrast, the C in TNC generally decreased as the amount of nitrogen supplied from fertigation was enhanced (Figure 5B). Applying foliar urea significantly increased (P<0.0001) the C concentration in the total amino acids at each given fertigation concentration, but significantly decreased (P<0.0001) the carbon concentration in TNC to a similar level across all fertigation treatments (Figure 5A, B). In addition, the concentration of total amino acids was negatively related to TNC concentration (Figure 6), with a 1 mg g^{-1} increase in amino acid concentration resulting in a decrease of approximately 2 mg g^{-1} of TNC. These results, therefore, demonstrate that the synthesis of amino acids and proteins for storage in almond trees occurs at the expense of carbohydrates.

In conclusion, young almond trees accumulate nitrogen dynamically in both the amino-acid and protein forms, which are closely related to N supply. Enhancing the amount of available nitrogen through either fertigation during the growing season or foliar urea applications in the fall increases the concentrations of both free and total amino acids in storage. Although the proportion of nitrogen stored as free amino acids increases as the N supply increases, we believe that protein is still the primary form of stored N in dormant young almond trees. Arginine, the predominant amino acid in both free and total amino-acid pools, accounts for an increasing proportion of the pool as the nitrogen supply increased. However, the proportion of arginine N in the free amino acids is higher than that in the total amino acids. In addition, the synthesis of amino acids and proteins occurs at the expense of carbohydrates, and the amount of TNC used for N assimilation increases as the supply of nitrogen increases.

This research was supported in part by the California Fruit Tree, Nut Tree, Grapevine Improvement Advisory Board, and by USDA-ARS. The authors thank Priscilla Licht for editorial assistance.

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